

FINAL TECHNICAL REPORT for NASA-AMES AGREEMENT No. NAG 2-26

1. Vestibular Compensation

a. Neurophysiological studies on MVN neurons during vestibular compensation. Second and higher order horizontal-canal related MVN neurons dynamic response properties to natural stimulation in the form of horizontal angular head acceleration were examined in compensating gerbils. In addition, these studies provided data on the influences of non-vestibular sensory input to the MVN that may provide a mechanism for both the regulation of spontaneous activity and dynamic responses to head rotation via spinal cord input to the MVN.

A series of experiments concentrated on three problems related to vestibular compensation; the identity of "plastic" neurons in the recovery of the VOR, the role of vestibular commissural interconnections in compensation, and the mechanism by which ascending spinal pathways facilitate compensation. Decerebrate gerbils (with the cerebellum intact) were studied under five different conditions: normal (LI), acutely labyrinthectomized (AL) i.e. < 12 hours post-operatively, compensated 4 to 7 weeks post-operatively, LI T₂ spinal cord transected, and chronically labyrinthectomized T₂ spinal cord transected (decompensated). In all animals, electrodes were placed in the injured labyrinth and, in some instances, on the oval window of the intact labyrinth. Recordings were made from 345 type I and 308 type II MVN neurons bilaterally. Spontaneous activity, responses to ipsilateral electrical stimulation, and dynamic responses, to two frequencies (1.3 and 0.13 Hz) of horizontal sinusoidal head rotation, of type I and II neurons were measured.

Acutely following hemilabyrinthectomy, the few type I neurons that were detected ipsilateral to the injured side demonstrated severely depressed spontaneous activity and response gain (impulses/s/deg/s). On the contralateral side, type I neurons were plentiful and exhibited spontaneous activity not significantly different from LI gerbils. The average response gain was significantly depressed on the intact side at both frequencies, but was significantly greater than on the injured side. In compensated animals, the mean response gain was increased on the injured side versus in acutely hemilabyrinthectomized (HL) animals, but the mean response gain on the intact side was unchanged, such that, at both frequencies tested, gains were equivalent bilaterally. The gains remained low even when the activity rate of silent or slowly firing neurons on the injured side was artificially increased by galvanic polarization of the ipsilateral nerve. Thus, loss of spontaneous activity alone does not explain the insensitivity of type I neurons acutely after HL. Following vestibular compensation, both the number of type I neurons on the injured side and their spontaneous firing rates increased. However, neither measure attained the values demonstrated in the normal animal or in the contralateral MVN. On the intact side, the spontaneous rate also increased with compensation. Thus, although the spontaneous activity of type I neurons on the ipsilateral side was improved versus the acute HL condition and the number of active neurons was greater, there remained an asymmetry in type I neuron spontaneous firing rates and bilateral distribution across the midline in the compensated animal. The increase in firing rate of type I cells on the intact side was coincident with a reduction in the number of these neurons which were silenced (rectified) during any portion of the stimulus cycle at 1.3 Hz. Thus, it appears that the function of the increase in the spontaneous rate on the intact side is to increase the linearity of the neuronal responses at high frequencies. In these experiments, compensation with regard to type I MVN activity involves a restoration toward bilaterally symmetry and linearity in gain while restoration of normal response phase, response gain, and symmetry in spontaneous activity and number of active cells did not occur. This is similar to changes seen in the HVOR during compensation in primates (15) and cats (27). In the cat, the HVOR is asymmetric, of low gain, and phase advanced (27). With time, the response becomes symmetric but does not recover full gain and remains phase advanced. Thus, it appears that during compensation, dynamic type I neuronal activity in the MVN correlates with recovery in the HVOR and suggests to us that compensation in the HVOR is the result of a mechanism acting at type I neurons.

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The role of commissural mechanisms in the modification of MVN type I responses was inferred from studies of an interneuron involved in this pathway, the MVN horizontal canal-related type II neuron. The spontaneous activity of the type II neurons was not significantly changed on either side of the midline in the acute HL animals. However, the gain was depressed bilaterally, more on the intact side. With compensation, as in the type I neurons, the gain becomes symmetrical bilaterally but depressed compared to normals. However, the number of type II neurons on the intact side in the AL animal is reduced and remains so in the compensated animal. It appears that while some type I neurons on the injured side regain activity with compensation, this does not effectively restore the commissural system to type I neurons on the intact side because there remain type II neurons. The commissural pathway from the intact to the injured side is also somewhat depressed. The increase in spontaneous activity necessary to preserve linearity in the type I responses could result in suppression of the type I activity on the injured side. However, the efficacy of this pathway must be preserved, since it is responsible for the modulation of type I neurons on the injured side in response to head movement. We also feel that the gain of the type I response ipsilateral to the lesion is not a result of increased commissural input since the gain of the ipsilateral type II neuron is not significantly changed with compensation. Thus, the commissural system may be necessary for modulation of type I neurons ipsilateral to the peripheral ablation but cannot be solely responsible (sufficient) for compensation. Other factors must account for the increase in type I spontaneous activity bilaterally and the improvement of type I gain on the intact side. These may include input from a non-vestibular source(s) that contribute(s) to both spontaneous activity and responses to head acceleration. Reports on these findings have been published.

b. Effects of spinal cord lesions on VNC neurons during compensation. LI and compensated gerbils were compared following acute transection of the spinal cord at the T₂ segment to assess effects of such lesions on spontaneous activity and dynamic responses of type I and type II MVN horizontal canal-related neurons. The level of spinal cord transection was intermediate to that used in two different sets of studies on compensated guinea pigs where lesions at approximately T₁₂ (2) or unilaterally in the ventral funiculus at approximately C₁, produced reversal of the responses of VNC neurons to head tilt and also produced postural/oculomotor decompensation (2,21). The T₂ lesion did not compromise respiration although a gradual reduction in mean arterial blood pressure occurred over a course of 6 hours post-transection in both LI and HL preparations. The data obtained from those animals were pooled for statistical comparison with that of the studies described above. Average spontaneous activity rates, response gains at two frequencies of horizontal head rotation and response phase measures were compared. In addition, an analysis of variance was performed comparing each set of samples obtained in separate electrode tracks through the MVN to determine if sample bias distinguished each experimental group. This latter set of comparisons was non-significant as were interactions between the experimental variables and the sample time during the recording session. In LI cord damaged gerbils, the percentage of type I and type II neurons was comparable to that obtained in LI cord intact controls. Spontaneous activity rates were also statistically similar between the two groups. Response gains however, were reduced for both types of neurons at a rotational frequency of 1.3 Hz. For type II neurons, gain was reduced for slower head rotation (0.13 Hz). Response phases were unaltered. In compensated HL cord damaged animals, a bilateral asymmetry in the distribution of both types of cells was found that was comparable to that observed in compensated HL cord intact animals. The activity of type I neurons ipsilateral to the side of the labyrinth ablation was significantly lower than in the cord sectioned LI group, while on the contralateral side, type I discharge rates were unaltered. Similarly, type I response gains were reduced compared to LI cord damaged controls. A bilateral asymmetry in type I gain was found (contralateral side greater than the ipsilateral) for the lower rotational head frequency. At the higher frequency of rotation, gains were reduced but bilaterally similar. As in AL gerbils, bilateral gain asymmetries were induced by the added spinal cord damaged in compensated animals. Response phases remained asymmetric, with a greater phase lead in the responses of type I neurons on the side contralateral to the labyrinthectomy. In contrast to type I cells, type II neurons spontaneous activity rates were not significantly different from those of LI cord transected gerbils. These findings suggest that ascending spinal cord input may exert a marginally excitatory influence for both types of cells in the LI preparation while facilitating their dynamic response

to head rotation. The mechanisms underlying such effects were not assessed by these initial studies; however, both direct spinovestibular and polysynaptic pathways are probably involved. The effects of acute spinal lesions on MVN activity and dynamic responses differed between type I and type II cells. This finding argues against an interpretation based on non-specific effects due to spinal shock (46). The overall effect of the lesions is consistent with the behavioral observation that spinal cord damage can cause a partial reversal of vestibular compensation as evidenced by a reoccurrence of motor signs of HL. Our evaluation of the results is that the effects of cord transection are manifested both through a reduction in the performance of type I neurons and a reversal of synaptic efficacy in the commissural pathway from the intact side to the injured side, especially at the inhibitory connection between the type II neurons and type I neuron, both ipsilateral to the injured labyrinth. This is in part based on a closed loop model described below.

c. A closed-loop vestibular compensation model for horizontally canal-related MVN neurons. Several different schema have been proposed based on the functional connections between the bilateral MVN neurons as related to mechanisms underlying recovery of the VOR and bilateral activity rate and responses in the VNC during vestibular compensation. Galiana and colleagues (16) evaluated two different general models (closed and open loop) of commissural connections between the bilateral vestibular nuclei specifically concerning connections between type I and type II neurons. Favoring a closed-loop model, they sought to explain how alterations in the gain of signals transmitted across the midline, through the commissural system, could account for both re-establishment of bilateral symmetrical activity and responses and postural compensation of static head tilt for unilateral lesions and for serial lesions that produce Bechterew phenomena. This model was in part based on observations of the effects of serial labyrinth lesions on lateral head tilt in the HL frog (4). The model however was not supported by any additional direct experimental evidence concerning neuronal activity. In addition, by inference the model assigns a role for type I and type II MVN neurons in the regulation of HL-induced head tilt. This may however be a function subserved by otolith-related neurons in other pathways of the VNC (50). Moreover, the frog brainstem commissural system differs functionally from that of mammals. In addition, other commissural systems through the cerebellum may also be involved in compensation (14). We performed behavioral tests in gerbils of the Galiana model and failed to support its prediction concerning the effects of lesions of those pathways for both the development and the maintenance of postural compensation in that species. Other investigators have also failed to confirm the models prediction in the guinea pig (47). Additional critical evaluation was provided by Fetter and Zee (15) who demonstrated that gain changes in a closed-loop model were not required to account for improvements in VOR gain in compensated HL monkeys. They proposed a modification of that model using estimates of spontaneous activity rates at various stages of compensation in type I and type II cells related to the horizontal VOR. We have recently provided a further development of that model based on our experimental data on dynamic responses to horizontal head rotation in horizontal canal-related MVN cells. The equations originally formulated by Fetter and Zee (15) were altered to allow weighting factors associated with bilateral asymmetric distribution in the number of responsive MVN cells observed at different stages of compensation. In addition, we proposed an estimate of synaptic efficacy that is calculated as a ratio of gains across each set of connections between type I and type II neurons in the classic commissural circuit proposed by Precht et al (37,38). The most significant result of the application of our model is that the efficacy of the inhibitory connection between type II and type I neurons in the MVN ipsilateral to the injured labyrinth, increases three-fold in compensated animals as compared to LI gerbils. This change in efficacy especially occurs at a frequency of head rotation in the range of natural head motion (48). In compensated animals, the results of spinal cord lesions is an alteration in the ratio of gains so as to reduce synaptic efficacy. In those same preparations however, the synaptic efficacy in the direct commissural excitatory connection between contralateral type I and ipsilateral (injured side) type II neurons is unaffected. The model and further discussions of its implications are contained within the published (Newlands and Perachio; Exp. Brain Res. 82:373-383, 1990; Brain Res. 541:129-133, 1991) papers.

d. Spatiotemporal convergence in VNC neurons. We have demonstrated in decerebrate rats that a significant percentage of horizontal canal-related type I and type II neurons are responsive to dynamic

linear head acceleration. Two distinct classes of neurons were identified that demonstrated spatial/temporal characteristics in their responses to translational motion along vectors that lay parallel to the earth horizontal plane and that were directed through the horizontal head plane. For both types of responses, gain was a function of vector orientation and response phase varied as a function of vector angle. Vectors of maximum sensitivity (S_{\max}) were oriented at an angle of 90° from the vector of minimum sensitivity (S_{\min}). Among those horizontal canal-related neurons for which S_{\min} was oriented 90° counterclockwise from S_{\max} where S_{\min} responses temporally lead S_{\max} responses, the response characteristics of type I and type II neurons were statistically similar. There was also a highly significant linear correlation between the magnitude of the response to both vectors. We propose that such neurons are connected by a commissural pathway that is organized as a closed-loop; therefore, both canal and otolith-related signals are carried via commissural fibers. Among cells for which S_{\min} is oriented 90° clockwise to S_{\max} , type I and type II cells differed in their responses to linear head acceleration. We proposed that these latter types of neurons are organized as part of an open-loop commissural system. Therefore, the otolith input to type II neurons for that system is proposed to be derived from the ipsilateral labyrinth. We further propose that the closed-loop system is related to the velocity storage mechanism. It has been demonstrated that commissurotomy or HL damages velocity storage in primates (22). An open-loop commissural system would clearly be less vulnerable to commissurotomies since both otolith and canal-related signals would be available bilaterally for both types of HC cells. In HL animals, velocity storage does not recover to normal levels (15). We predict that, following acute HL, HC-related cells with counterclockwise responses will not be detected and clockwise responses will remain. In compensated animals, some degree of recovery may occur in the counterclockwise population to support the partial restoration of velocity storage-related functions. An analysis of the frequency dependent characteristics of the responses of cells with two-dimensional sensitivity to linear acceleration reveals functional relationships for the responses at the two orthogonal vectors. For HC neurons, the ratio between the response gain for S_{\min} relative to S_{\max} is smallest for the lowest tested frequency (0.14 Hz) and increases with frequency. This was due to a relatively small S_{\max} gain across frequency while S_{\min} gain increased ten-fold over a decade of stimulus frequency. This finding along with the observation that response phase for S_{\min} temporally leads that for S_{\max} by 90° , defines a derivative relationship between the responses at the two orthogonal vectors. In terms of the relationship to the stimulus, therefore, the S_{\max} response is in phase with peak linear acceleration whereas S_{\min} responses appear to be related to its derivative or the rate of change of linear head acceleration (jerk). The response vectors for HC cells were distributed for the test sample throughout the horizontal head plane.

Vertical canal-related (VC) neurons differed in their responses to linear acceleration from HC neurons. As frequency increased, S_{\max} gains decreased and S_{\min} responses were low and unchanging with frequency. S_{\max} vectors were oriented ipsilaterally and tended to align with the vertical canal planes in the horizontal head plane. The response properties of HC and VC cells with convergent otolith organ inputs were consistent with the spatio-temporal transformations that must be performed in the vestibulo-ocular pathway to generate horizontal, vertical and torsional eye movements that are produced by linear head acceleration in the rat (19). Linear translational motion produces compensatory eye movements that require high pass filter characteristics of the underlying neuronal system. These characteristics are exhibited by HC related convergent input cells for S_{\min} vectors. Otolith-ocular responses to static head tilt consist of vertical and torsional eye movements with low pass filter characteristics. VC cells responses to S_{\max} vectors exhibit such characteristics. Finally, the broad frequency response range of convergent input neurons to linear acceleration is compatible with the required signals to support the enhanced VOR induced by coacting linear and angular accelerations at both low (41) and high frequencies (42).

e. Contributions of irregularly firing vestibular afferents to linear and angular VORs. Two studies were conducted that were based on findings of the existence of canal otolith two-dimensional neurons. Spatiotemporal characteristics of those cells were modeled based on the assumption of convergent inputs from both regular and irregularly firing afferents. It had been demonstrated by Minor and Goldberg (30) that silencing of irregularly firing afferents by application of anodal currents bilaterally

across the labyrinths of alert squirrel monkeys did not affect to mid-range frequency HVORs. The tonic/phasic characteristics of the responses of irregular otolith afferents; however, could contribute to the steady state component of nystagmus produced by off-vertical axis rotation (OVAR). This vestibulo-ocular response has been attributed to inputs from the otolith organs and also requires the central velocity storage mechanisms. In three squirrel monkeys, we demonstrated that application of anodal currents, sufficient in strength to silence irregularly firing primary afferents, reduced or abolished the otolith ocular response to OVAR. This was not attributable to disruption of the velocity storage mechanism since comparable stimulation during optokinetic nystagmus (OKN) or optokinetic after-nystagmus (OKAN) was not effective. On the basis of those findings, we propose that irregular afferents are required to produce the compensatory slow phase eye velocity that matches angular head velocity during OVAR. We further propose that the underlying mechanism is related to the spatiotemporal convergence of regular and irregular otolith afferents onto neurons in the VNC to produce two-dimensional spatial and temporal responses to linear head acceleration.

We used the same test paradigm to duplicate the findings of Minor and Goldberg (30). However, we also demonstrated that functional ablation of irregular afferents with translabyrinthine anodal DC currents does have an effect on the HVOR to horizontal velocity steps. Application of current during perrotatory nystagmus transiently reduced the slow phase eye velocity; however, the time constant of the response was unaltered. We suggest that irregular afferents contribute to the generation of the VOR to low frequency head rotation possibly through central neurons with low pass filter characteristics. In the proposed studies, we will employ both anodal and cathodal galvanic stimulation to assess the effects of functional ablation or excitation of primary afferents of the intact labyrinth and of excitation ganglion cells on the side of ablation in HL animals on vestibulo-ocular responses.

f. Application to flight studies. The electrode implantation technique implemented for the squirrel monkey was modified for use on another project funded by NASA (NAG 2-446). Stimulation electrodes were implanted in the perilymphatic space near the round window of rhesus monkeys to provide a method for electrophysiological identification of second order neurons in the VNC. The animals were prepared for studies conducted in conjunction with a joint NASA/Russian sponsored orbital flight experiment (COSMOS 2229/BION 10). The experience gained in our work related to the present grant, thus, contributed directly to successful application in a space science project.

g. Metabolic measures in vestibular neurons. Spontaneous activity of vestibular afferents depends on the integrity of their connection and the viability of the sensory hair cells of the vestibular neuroepithelium. Following extirpation of the sensory end organs, spontaneous activity in the afferents is reduced to virtual silence (46); however, the neurons remain sensitive to activation by direct electrical stimulation (31). Vestibular compensation results in increased activity in the deafferented ipsilateral VNC (31) and near total restoration of the vestibulo-ocular function (15); however, afferent activity is not restored. The mitochondrial enzyme, cytochrome oxidase (CO), which is involved in neuronal oxidative metabolism, has been found to vary as a function of neuronal activity (49). Histochemical staining for CO has been used to illustrate changes in central vestibular neurons in the auditory (20) and visual system (49) following loss of primary afferent input. We have used CO histochemistry and hemilabyrinthectomized gerbils to assess changes in metabolic activity in the primary afferent neurons of Scarpa's ganglion and in the MVN at various time points postoperatively. We anticipated that, since the neurons associated with the damaged labyrinth do not die and do not exhibit discharge activity, metabolic demand should be greatly reduced resulting in decreased CO staining. Animals were sacrificed at 1 hour, 72 hours, 2 weeks, 1 month, and 3 months post-HL. Quantitative measures were made of CO staining using microdensitometry on samples of 50 ganglion cells per side in each animal. At 1 hour postoperatively, an asymmetry was apparent bilaterally in CO staining intensity. This asymmetry became statistically significant by 2 weeks and remained so for the samples taken after longer survival. Thus, metabolic changes lag the decrement in neuronal activity induced by the lesion. In the VNC, it was more difficult to obtain statistical samples since the cell bodies are smaller than ganglion cells and the neuropil also was markedly stained. We, therefore, obtained areal measures bilaterally from regions in the MVN that we had previously determined, in retrograde labeling studies,

contained concentrations of vestibulo-ocular neurons. Asymmetry in CO staining were detected in samples from groups that survived hemilabyrinthectomies by two weeks or more. Finally, we obtained samples from gerbils that have developed spontaneous fatty tumor growth in the middle ear cavity (cholesteatomas). Such tumors, clinically, are detected by inner ear dysfunction. The ipsilateral MVN was found to exhibit extensive loss of CO staining, especially, in the rostral pole of the nucleus that is rich in vestibulo-ocular neurons.

h. Immediate early gene expression following vestibular stimulation. A class of gene that is expressed relatively rapidly and for transient periods following stimulation of specific neuronal systems has been defined as immediate early genes (13). Following sensory stimulation, immediate early gene-related (IEG) transcription factors, such as the Fos protein are induced in neurons in central sensory pathways after either natural or electrical stimulation or lesions of the sensory apparatus. Dr. Galen Kaufman, who joined our laboratory this year, has demonstrated immunohistochemically evidence of Fos expression in the VNC, inferior olivary complex, prepositus nucleus and other brainstem areas in the rat brain following constant centrifugal acceleration in the horizontal head plane (23). Expression of transcription factors requires a period of time following stimulus onset and regresses within hours of the sensation of the input and thus is not a simple reflection of neuronal activity. Although for most cases, the physiological consequences of this primary gene response have not been defined, IEG protein expression is thought to be involved in the regulation of subsequent gene-related activity that is involved in cell control and signaling.

In our preliminary studies, we first confirmed that Fos expression could be induced in the brainstem neurons of gerbils in a fashion comparable to that previously described by Kaufman and his colleagues for the rat. We first determined that Fos induction could be achieved within less than 30 minutes of centrifugal acceleration by off-axis rotation at constant velocity (360°/sec.) which was one third less time than previously reported. The neuronal groups that stained for Fos were identical for those found in the rat. Thereafter, we initiated a series of experiments employing electrical stimulation of the labyrinth to elevate or selectively reduce afferent input. The purpose was to define a stimulus that could be applied selectively to separate afferent nerves and that could be controlled in a functionally bipolar fashion.

Application of anodal current resulted in a silencing of irregularly discharging afferents with DC currents as low as 30 μ A. Regularly firing afferents were never silenced even with increases in anodal current greater than an order of magnitude. Current was applied for 30 minutes. In some instances, we recorded from the same afferent throughout the period of stimulation. Periodically, current was turned off to confirm the integrity of the cell evidenced by its discharge activity and sensitivity to head rotation. In a single instance, we observed the cell that did not exhibit normal activity following 20 minutes of stimulation; however, activity comparable to that recorded pre-stimulus could be restored by application of cathodal current. In our initial experiments, we utilized gerbils that were anesthetized with urethane or ketamine. These anesthetics alone produced Fos activation in viscerally related neurons under urethane and in the VNC and inferior olivary complex with ketamine. Subsequently, using methods comparable to those employed in our squirrel monkey preparations, we have conducted our experiments on chronically implanted gerbils that were unanesthetized at the time of stimulation. In cases where 100 μ A of anodal current were applied for 30 minutes, a pattern of Fos labeling was found in the medial vestibular nuclei, prepositus nucleus and contralateral beta nucleus of the inferior olive (see Figure 1). The latter site did not express Fos with sustained stimulation in anesthetized animals perhaps due to blockage of polysynaptic transmission. Cathodal DC stimulation produced Fos immunostaining in the same nuclear areas. In addition, Fos was expressed in neurons in the abducens nuclei. The stimulus was applied across the entire labyrinth. In the proposed studies, we will employ different electrode arrangements in order to produce specific sites of activation. Parametric studies will be designed to determine the time course of IEG responses. We will also examine other IEG transcription factors. In addition to their role in regulation of cell activity, IEG factors have also been implicated in neuronal plasticity (44). We have used antibodies to c-Jun and the zinc finger protein zif 268 (also known as NGFIA, egr1, TIS8, krox 24 and d2). Depending on the stimulus, these factors may be expressed with

the same or different time courses or with differential sensitivity. In our preliminary studies, we have examined zif 268 immunoreactivity following hemilabyrinthectomy and electrical stimulation of the labyrinth (Fig. 2). Those data have not to date been completely analyzed; however, we have determined that specific groups of neurons in the VNC and granular layers of microzones of the vermal cerebellar cortex exhibit zif 268 immunoreactivity following hemilabyrinthectomy.

Figure Legends

Fig. 1. Fos immunolabeling (IL) in the medial vestibular nucleus (MVe) and prepositus (PrH) of the alert gerbil following thirty minutes of 100 μ A unilateral cathodal polarization of the labyrinth. Nuclear immunostaining for the protein appear as a dense black reaction product in this computer captured image of a transverse section through the brainstem.

Fig. 2. Zif 268 immunolabeling in the prepositus nucleus of the gerbil six hours following hemilabyrinthectomy.

Fig. 3. Intracellular HRP labeling of vestibular afferent.

A. Schematic drawing of the projection and termination sites of an intracellularly labeled utricular afferent. INSET: Horizontal section through the decalcified labyrinth and brainstem of the gerbil at the level of the utricle.

B. Photomicrograph of a whole mount of a gerbil saccule illustrating a dimorphic ending (calyx plus bouton terminals) of an HRP labeled afferent.

C. Central projections and terminals of an HRP labeled horizontal canal afferent axon. Terminal field and bouton-like endings of an irregularly firing canal afferent are shown in the superior vestibular nucleus.

Fig. 4. Time course of retrograde pseudorabies virus labeling of olivo-cerebellar cells in the gerbil. Top photo: Few cells are labeled in the beta nucleus of the inferior olive (IO) one day following midline injection of PRV into the uvula. Middle photo: Extensive labeling of neurons in the beta nucleus 30 hours post-unilateral uvula injection. Bottom photo: Bilateral labeling of I.O. 46 hours post unilateral uvula injection. Primary labeled cells are vacuolized and nonneuronal labeling is extensive in the contralateral IO.

Fig. 5. Vestibular and oculomotor-related behavior of cells and squirrel monkey brainstem during optokinetic nystagmus. Top photo: Response of a VNC cell, in an alert squirrel monkey, to interaural axis linear acceleration at peak acceleration $\pm 0.15 \times g$. Upper trace: head position, Lower trace: extracellular recordings of action potentials. Bottom photo: eye position/pause type brainstem neuron in an alert squirrel monkey. Upper trace: horizontal eye position. Middle trace: vertical eye position. Bottom trace: action potentials

Fig. 6. 3-dimensional reconstruction and analysis of vestibular efferents. Examination of the utricular macula contralateral to the site of the extracellular injection of biocytin reveals wide spread labeling of terminals in the sensory neuroepithelium. (A) Top view of the macula with a 3-dimensional reconstruction of two separate labeled cells. The photograph in the *inset* shows a portion of a labeled terminal field with multiple boutons-en-passant. The asterisks in this and the remainder of this figure indicate the cut end of the axon of an efferent neuron, innervating the striolar region, as it enters the utricular macula. (B) Orthogonal view and (C) A 22nd order dendrogram generated from arbor analysis of the central terminal field.

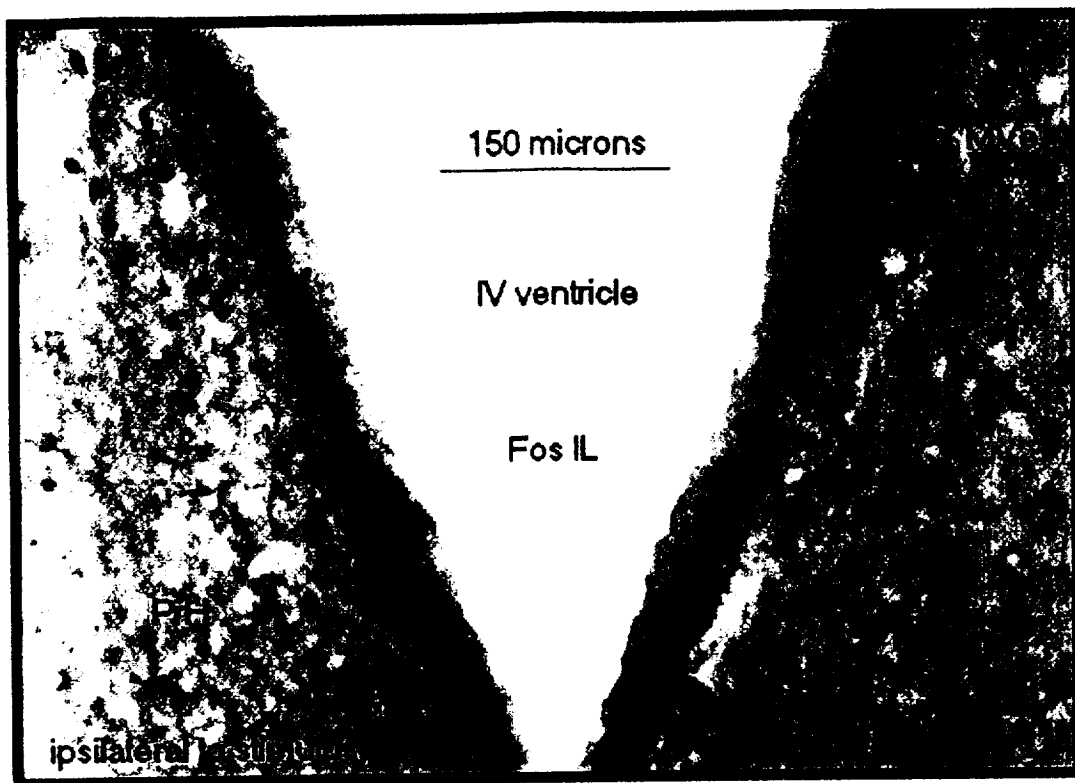


Figure 1

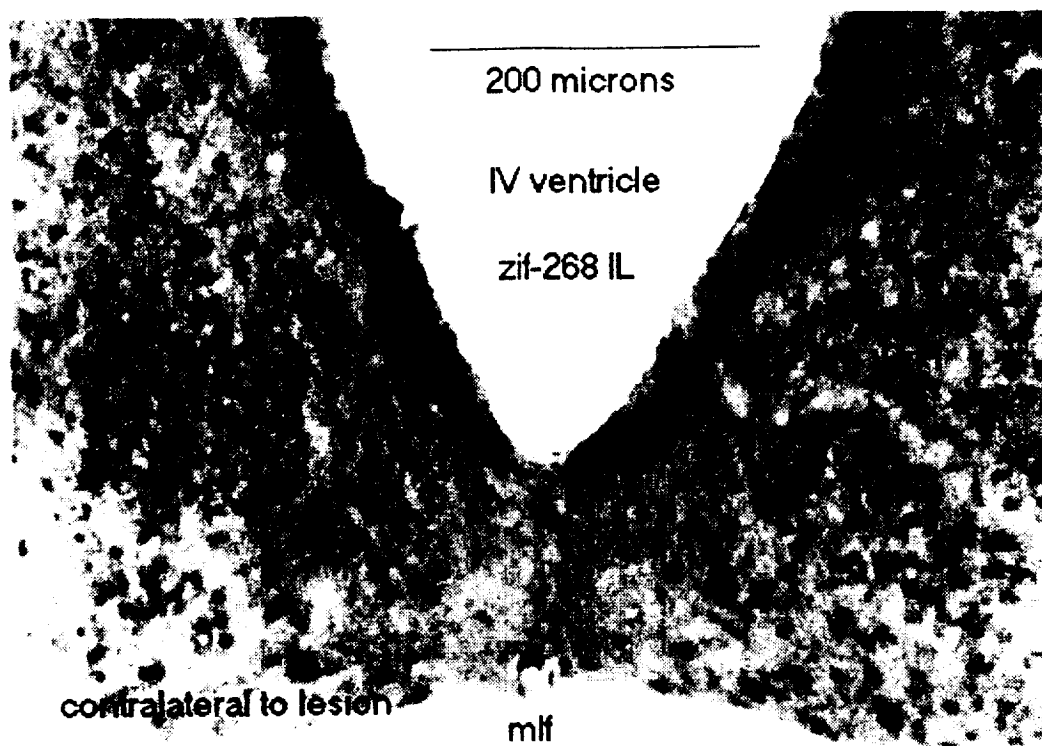
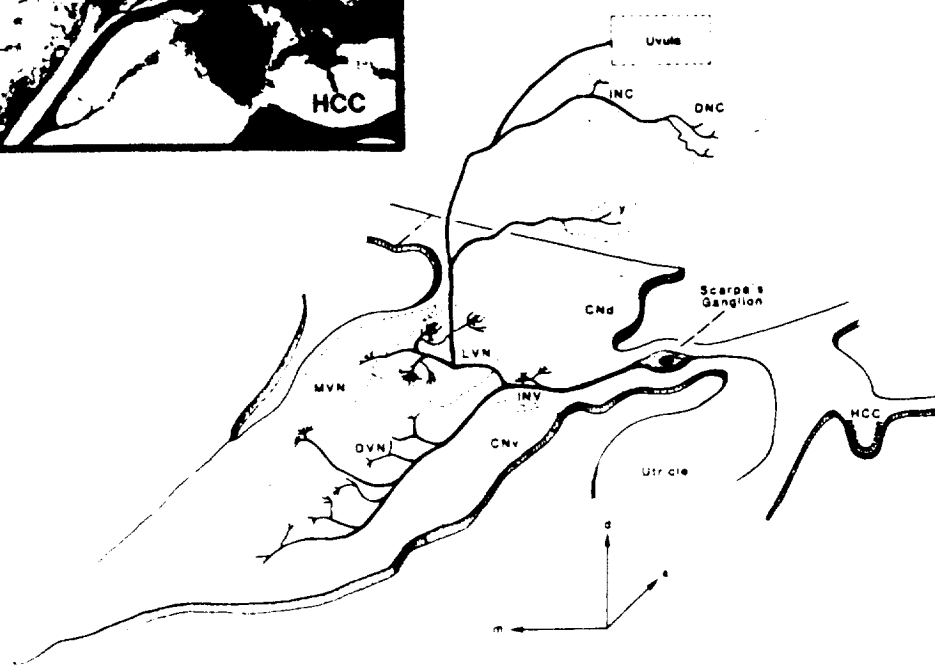
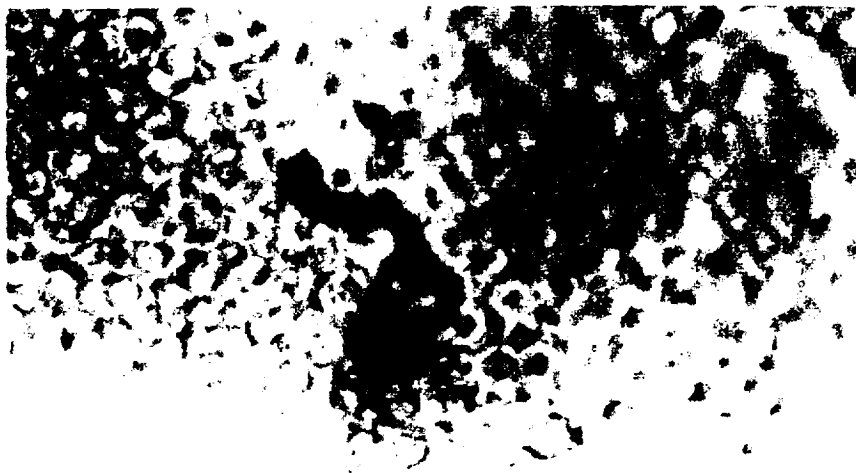


Figure 2

A.



B.



C.



PRV infection progression
from uvula (~200 nl)

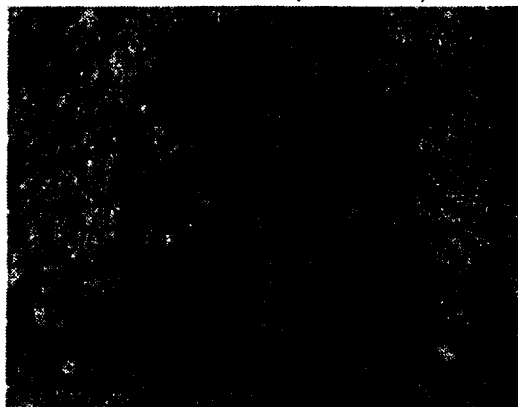


Figure 4

$Y^+, 0.7\text{ Hz}$

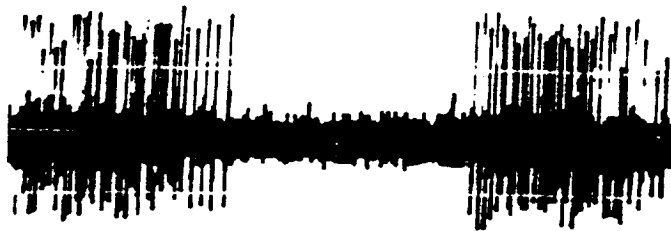


Figure 5

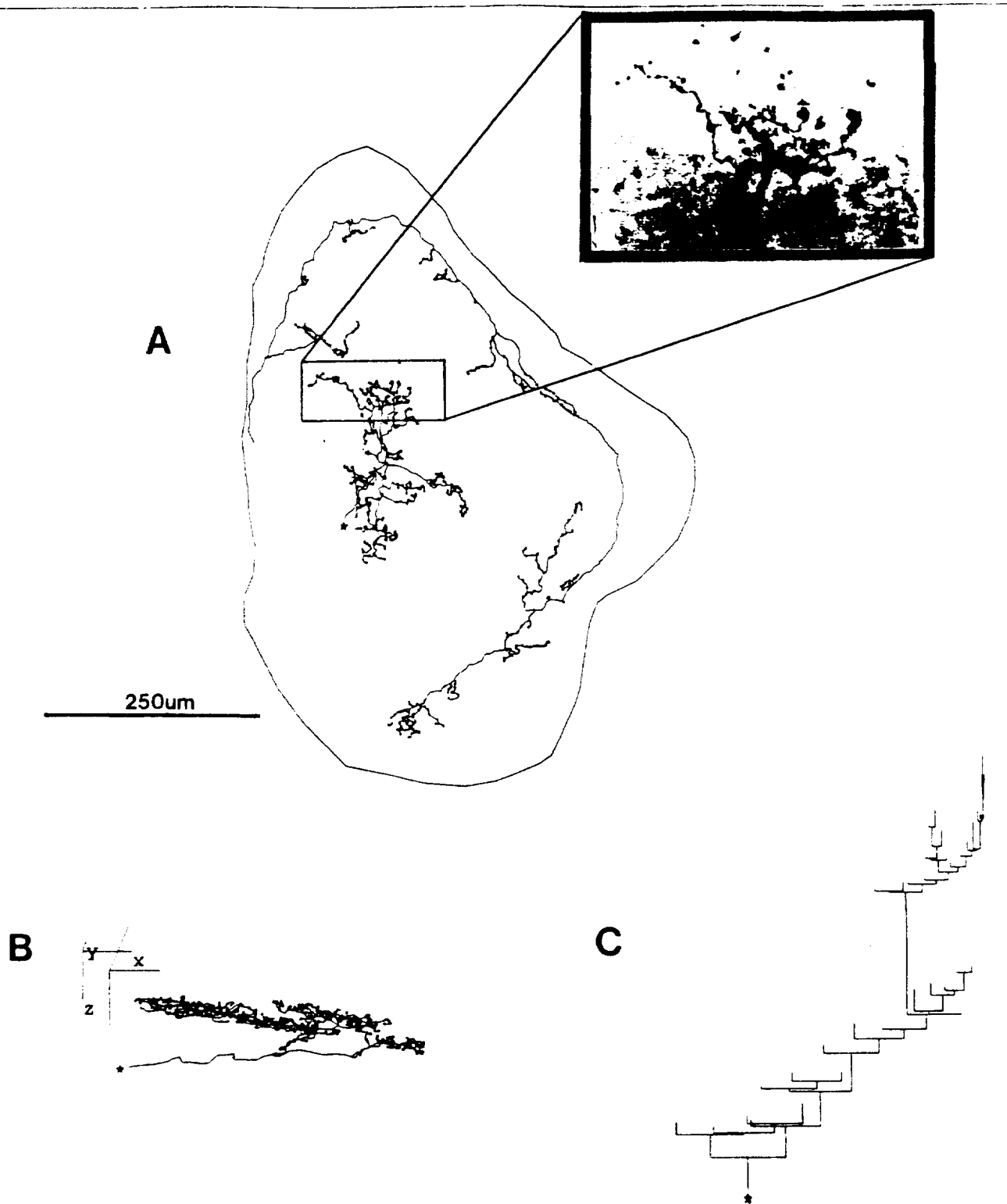


Figure 6

i. Morphological studies. Primary afferents: In one set of experiments, we have employed intracellular labeling techniques to inject horseradish peroxidase into physiological identified primary afferent neurons. The histological procedures involve serial sectioning through the brainstem and cerebellum to allow us to trace the course of the labeled central axon through its arborizations to terminal sites in the VNC and cerebellar cortex. For collaborative work with Dr. M. Ross of the NASA/Ames Research Center, we also prepared the end organs of the sensory neuroepithelium to examine the peripheral labeled processes and their terminals on sensory hair cells. In most cases, we have successfully traced the central projections; however, peripheral tissue has been less reliably labeled. More recently, we have improved our yield by increasing injection time and by examination of the end organs after clearing and embedding. We have found that the latter procedures allow visualization of the thin processes of the axons that may lie deep within the tissue. The embedded tissue has been taken to the Ames laboratory for serial sectioning and for three-dimensional reconstruction. Another impediment to this work has been our limited ability to functionally characterize otolith organ related afferents during intracellular recordings. This was due to microvibrations that were produced by the metal bearing on metal linear rail rolling contact on the linear track. During the past six months, we have designed and constructed a new track based on an air bearing slide that rides on a precision surface granite slab. The air bearings eliminate stiction and rolling friction, both of which contributed to our problems maintaining intracellular penetration of primary afferent axons during dynamic linear acceleration.

We have proceeded with serial three-dimensional construction of central axons of labeled afferents. The typical pattern we have observed in virtually all irregularly firing afferents is an extensive arborization of the axon after it penetrates the brainstem medial to the restiform body. Three major branches derive from the parent axon (see Fig. 3A). An ascending branch sends projections to the superior vestibular nucleus (SVN), rostral medial vestibular nucleus (MVN) and rostral ventral lateral vestibular nucleus (LVN). A collateral from this major daughter axon projects dorsally to innervate accessory nuclei, such as nucleus γ for saccular afferents, the nucleus interpositus of the cerebellum and the granule cell layer of the cerebellar cortex in lobules I, IX, and X. The descending branch collateralizes from the main axon, at approximately right angles to the ascending branch, and courses caudally parallel to the midline. At intervals, medially directed collaterals are derived from the descending branch to innervate the middle and caudal portions of the MVN, the caudal ventral LVN and the descending vestibular nucleus (DVN). In the terminal fields in each nucleus, extensive minor branching is seen. Varicosities may be observed along the length of the same axon suggesting terminals en passant. Most frequently, axon branches end in a cluster of bouton-like endings (see Fig. 3B). Multiple branches into the VNC are also labeled in intracellularly injected regularly firing axons. The parent axons of this class of afferents is significantly thinner than that of irregularly firing cells; therefore, they are both more difficult to penetrate and to inject successfully. Our impression is that the projections in terminals are fewer in number for irregularly discharging afferents. A similar finding has been reported for horizontal canal-related afferents of the cat (43). In proposed studies, we will continue this work to obtain a statistical sample of both canal and otolith organ-related afferents.

Central vestibular pathways:

A second set of morphological studies was begun this year. In that work, strains of α -herpesvirus, the Bartha and Becker strains of pseudorabies virus (PRV), were used to label vestibular afferent and efferent pathways (9,10). Those viruses have been used to map a variety of neuronal pathways. Like other herpes viruses, PRVs have an affinity for neurons. The virions are taken up by neuron terminals and transported retrogradely, or following injection into the vitreous fluid of the eye, anterogradely. They are replicated in the cell bodies and transported transynaptically. Recent evidence has been reported indicating that the virus is confined to specific passage through sites of synaptic connection between neurons rather than at contact points of cell fusion or through extracellular release. Spread of infection appears to be regulated by non-neuronal (e.g., glial) cells (8). The two viral strains are transported differently through the visual system of the rat. The Becker or wild strain appears to be

more virulent and transports faster and more extensively. The Bartha strain is attenuated and is less virulent and appears to be transported in different subsets of the neurons in the visual pathway.

We are using these PRVs to label central neurons related to the vestibular periphery and to trace central pathways that are putatively involved in connections between the visual and vestibular systems. We have established a Biosafety Level 2 containment facility in the laboratory, that was approved by the UTMB Environmental Health & Safety office. Injected animals are maintained in that confinement area from the time of injection until they are sacrificed by anesthetic overdose and perfused with mixed aldehydes, transcardiac, to inactivate the virus and preserve the brain tissue. The virus is obtained from Dr. L.W. Enquist of the Division of Viral Diseases Research, DuPont Merck Pharmaceutical Company, Wilmington, DE. The antibody used in histochemical identification of viral infected cells is provided by the same source. Dr. Kaufman received training in the use of these viruses in the laboratory of Dr. R.R. Miselis, Department of Animal Biology, University of Pennsylvania Veterinary School.

We have determined that PRVs are effective in producing neuronal infection and are transported transynaptically in the vestibular pathways in the gerbil. Thus far, we have found that the attenuated or Bartha strain of the virus is transported primarily in a retrograde fashion following injection into the vestibular labyrinth. When the same strain is applied into the central nervous system, transport also appears to follow a retrograde direction. In Fig. 4 is illustrated the time course of retrograde labeling of the contralateral inferior olivary complex following PRV injection into the uvula lobule (IX) of the gerbil cerebellar cortex. Within 24 hours of inoculation, dense labeling of the somas and primary dendrites of cells in the β nucleus of the inferior olive is noted. Cellular morphology appears to be intact and no invasion of immunoreactive product is seen extracellularly. After an additional six hour survival, spread is seen into non-neuronal elements and some cells appear to be vacuolized. Labeled cells are also detected in other nuclear areas including the VNC. Following two days survival, extensive transneuronal retrograde label is observed. Cells in the olivary complex are markedly damaged. Retrogradely labeled neurons appear in known visual related cells of the accessory optic system that provide input to the β nucleus of the inferior olive (45). These include the dorsal and medial terminal nucleus and the nucleus of the optic tract. In addition, with longer survival times, labeling is observed in accessory ocular motor nuclei, in pontine nuclei and in the reticular formation perhaps all reflecting inputs to the VNC neurons from groups that are three or more synapses retrograde from the injection site.

Control studies were conducted involving injections to stomach lining and into the eyes of both rats and gerbils to compare infectivity between the two species and to replicate the previous works of Drs. Miselis and Enquist. Gerbils tended to survive the infection for longer periods than rats suggesting a slower transport rate. We also have made injection into other lobules of the cerebellar cortex and found labeled neurons in known projection nuclei associated with those regions. Other injections have been made recently in the VNC and spinal cord to assess the usefulness of this tracing method to label afferents in the vestibular nuclei and to trace pathways that are associated with vestibulospinal system.

This technique holds promise for examination of polysynaptic connections to the vestibular system via transneuronal transport mechanism. This will be particularly useful for assessing the route by which neurons, that are responsive to vestibular stimulation, receive input from the classical vestibular pathways. Examples of such neurons are subsets of cells that exhibit IEG mediated expression of transcription factors following protracted stimulation of the vestibular system. Thus we would be able to examine the afferent input of the dorsal medial cell column of the inferior olive, which exhibits Fos expression following stimulation of the labyrinth with hypergravity, and which is retrogradely labeled by PRV injection into the cerebellar cortex. Using a series of survival times we plan to examine anew the afferents of the central vestibular system. We will employ different strains of the virus to assess the sensory pathways originating in the labyrinth. Finally, we will confirm specific monosynaptic connections that are indicated by PRV labeling using tracers that are not transynaptically transported.

Vestibular efferent projection to the vestibular end organs: The purposes of this study were to successfully label the efferent projections into the vestibular labyrinth and to describe the pattern of innervation between and within the various end organs. These experiments were conducted by Ian Purcell, an M.D./Ph.D. candidate who has been supported by a NASA Graduate Training Grant NGT 50748. Previous retrograde labeling studies in this laboratory examined the location, bilateral distribution, cell area, and number of brainstem vestibular efferent neurons in gerbil. These studies confirmed the bilaterally projecting neurons, located adjacent to the abducens nucleus known as group-e, as an optimal site of injection for anterograde labeling of efferent projections to the contralateral ear. A series of experiments was then performed to perfect the technique for anterogradely labeling (extracellular) the group-e axons and terminal arborizations in the periphery. Successful labeling of even the smallest ($<0.25\mu\text{m}$) terminal fibers was achieved with the use of biocytin while only limited transport was achieved with other markers such as horseradish peroxidase. Examination of the end organs was limited to the contralateral side because we observed that primary afferent fibers traveling near the extracellular injection site were often retrogradely labeled into the ipsilateral sensory neuroepithelium. The bilateral nature of the efferents and the fact that primary afferents have not been reported to cross the midline, make the contralateral sensory neuroepithelium an excellent candidate for analysis of efferent innervation. Light microscopic examination of transverse sections ($40\mu\text{m}$) through the brainstem at the level of the genu of the seventh nerve, revealed labeled efferent axons traveling from the site of injection (*group-e*), across the midline ventral to the fourth ventricle, along the vestibular nerve, and through both superior and inferior portions of Scarpa's ganglia. Examination of plastic embedded peripheral tissue revealed labeled axons bifurcating in the stroma and forming a massive collateral network upon entering the sensory neuroepithelium of all the end organs. The large number of identified preterminal and terminal processes tended to travel between the hair cell and supporting cell layer and appeared to make contact with both type II hair cell and type I calyx endings by multiple bouton-en-passant type swellings (see inset Fig. 6).

Three-dimensional (3-D) morphometry and imaging: Several individual efferent fibers have been reconstructed three-dimensionally, using the Neuroleucida system and arbor analysis performed. It was noted that individual fibers tended to be highly branched (22-28th order see Fig. 6c) and have a large terminal field; confirming the prediction that individual efferent neurons have a divergent pattern of innervation in the vestibular end organs. Quantitative analysis of any regional variations in efferent terminal density across the macula or crista as reported in other mammals has begun. Preliminary data suggest that bouton en-passant terminals appear denser in the peripheral than the apical area for the crista ampullares. Examination of individually reconstructed efferents suggest that fibers that innervate the striolar area in the utricular macula have a wider distribution of innervation than those that innervate the peripheral portion of the macula (Fig. 6a). The implications of these findings suggest that efferent neurons that innervate the vestibular sensory end organs possess an intrinsic functional diversity.

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Final Disclosure of Inventions Report:

There were no patents or inventions made.

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